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Bcl-2 family proteins function in an evolutionarily conserved pathway regulating programmed cell death. In an effort to delineate this cell death pathway, we undertook a novel functional screening approach. The pro-apoptotic Bax protein not only promotes apoptosis in mammals, but can also induce cell death when expressed in yeast. Based on this observation, we screened a human cDNA library for genes whose products protect *S. cerevisiae* against Bax-killing. Two human proteins, designated Bax Inhibitors -1 and -2 (BI-1 and BI-2), were identified during the screen that inhibit the death-inducing activity of Bax both in yeast and in mammalian cell line models. The deduced amino acid sequence suggests that BI-1 is likely an integral membrane protein with six potential transmembrane helices, and this was biochemically by showing that BI-1 was predominantly located in the detergent-enriched phase during Triton X-114 phase separation. We found that BI-1 is localized to intracellular membranes, similar to Bcl-2 and Bax. Moreover, BI-1 is able to interact physically with Bcl-2 but not Bax, as demonstrated both by in vivo cross-linking and by co-immunoprecipitation assays. Therefore BI-1 is a novel apoptosis regulator which functions in the Bcl-2/Bax pathway for programmed cell death.

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## (5) INTRODUCTION

Programmed Cell Death (PCD), also known as apoptosis, is a highly regulated process in which cells are actively involved in their own demise. Aberrant regulation of cell death will have pathological consequences and may cause various human diseases, including cancer. Central to the regulation of PCD are the Bcl-2 family proteins with multiple members identified in mammals. Some of the family members, like Bcl-2 and Bcl-$X_L$, function to inhibit apoptosis, whereas some others exemplified by Bax and Bak, function to promote or induce PCD (reviewed by Reed, 1994; Yang and Korsmeyer, 1996). Genetic studies in C. elegans have revealed three critical genes, *ced-3*, *ced-4,* and *ced-9*, that are involved in developmental cell death. Among these Ced-9 plays a "master switch" role in the regulation of programmed cell death (Henartner and Horvitz, 1994; Vaux et al., 1992). Ced-9 is highly homologous to Bcl-2 suggesting that the function of Bcl-2 family proteins may be well conserved during evolution. In support of this notion, the human *bcl-2* gene when expressed in C. elegans can rescue *ced-9* mutant worms (Hengartner and Horvitz, 1994; Vaux et al. 1992). Moreover, human Bcl-2 can also protect some yeast strains from oxidative injury (Kane et al, 1993) as well as function as a cell death blocker in Sf9 insect cells (Alnemri et al., 1982).

In addition to regulating programmed cell death in multicellular organisms, certain Bcl-2 family members also appear to play roles in either inducing or antagonizing cell death when expressed in two distantly related yeast species, the budding yeast *Saccharomyces cerevisiae* and the fission yeast *Schizosaccharomyces pombe*. The pro-apoptotic Bax and Bak proteins were found to induce cell death when exogenously expressed in yeast. Furthermore, Bax- or Bak- induced yeast cell death is not attributable to nonspecific toxicity caused by overexpression of a heterologous protein, since the anti-apoptotic members like Bcl-2 and Bcl-$X_L$ were able to protect yeast from Bax/Bak killing (Sato et al., 1994; Honada et al., 1995; Zha et al., 1996; Greenhalf et al., 1996; Jürgensmeier et al., 1997; Ink et al., 1997). Structural-functional studies of Bax and Bak suggest that Bax or Bak utilizes similar mechanisms to kill yeast and mammalian cells, since the same region of Bax/Bak (the BH3 domain) is critical to the pro-death activity of Bax/Bak in both yeast and mammals (Zha et al., 1996; Ink et al., 1997). The implication of these findings is that perhaps at least part of the PCD pathway may even exit in yeast, the observations that mammalian apoptosis regulators also induce or inhibit yeast death have raised the interesting question of when during evolution PCD has emerged, but most significantly have prompted the potential use of the powerful yeast genetics for the investigation of complicated issues of PCD in mammalian systems. In this respect, Bax-caused yeast cell death has been successfully exploited to study the structure-function relationship of the Bax protein (Zha et al., 1996).

As proposed in Aim#3 of the proposal, we took a yeast genetics approach to further dissect the Bcl-2/Bax cell death pathway in mammals. By taking this approach, we have identified two human genes, designated BI-1 and BI-2, that prevent the Bax-induced death of *S. cerevisiae*. Over the past year we have devoted most of my effort to the characterization of one of these genes, BI-1. Our findings are summarized here.

## (6) BODY

**Hypothesis:**

We reason that since Bax and Bcl-2 function in a similar manner in yeast as in mammalian cells, other proteins that functionally interact with Bcl-2/Bax, especially those proximal to Bcl-2/Bax, may also interact with Bcl-2/Bax similarly in yeast as in mammalian cells. This functional cloning approach, albeit new to the study of programmed cell death, has been extensively employed in addressing other biological issues in higher eukaryotes. An excellent example would be the identification of key cell-cycle regulators Cdc2 and Cdk2 kinases by cross-species complementation of a *cdc2* mutant in S. pombe and a *cdc28* mutant in S. cerevisiae (Lee and Nurse, 1987; Elledge and Spottswood, 1991).

## Experimental Procedures, Results and Discussion:

### Library screening.

In an effort to identify novel players participating in the Bcl-2/Bax-regulated programmed cell death pathway, we performed a functional screening in yeast. For this study, yeast strain QX95001 was constructed by transforming the Bax-bearing plasmid YEp51-bax into strain BF264-15Dau (Lew et al., 1991). YEp51-bax is a *URA3*-marked yeast high-copy plasmid containing full-length mouse *bax* cDNA under the control of the galactose-inducible yeast *GAL10* promoter (Zha et al., 1996). Cells of strain QX95001 were verified to die upon transfer from glucose to galactose-containing medium (to induce Bax expression, data not shown).

A human HepG2 cDNA expression library (Lew et al., 1991) was transformed into strain QX95001 and screened for Bax resistant transformants on galactose-containing solid medium. The screening procedures and outcomes are summarized in Figure 1. Initially 75 Bax-resistant colonies were obtained out of 1 million transformants. These yeast colonies were patched on galactose-containing solid medium as a secondary screen to verify their ability to overcome Bax toxicity. 17 colonies turned out to be truly Bax-resistant based on their ability to grow on galactose-containing medium during the secondary screening.

Bax-resistant colonies could result either from the presence of a human cDNA transformed into the yeast cell, or from a yeast mutation that suppresses the death-inducing activity of Bax. To distinguish between these possibilities, a standard "con-commitant - loss" assay was employed here. The rational is that if the Bax-resistance phenotype was caused by a library-derived cDNA , then the loss of the library plasmid (indicated by the con-commitant loss of the plasmid marker, *URA3* in this case) would result in loss of the Bax-resistance phenotype. On the other hand, if the Bax-resistance was caused by a yeast mutation, then the loss of the library plasmid should not have any impact on the Bax-resistance phenotype. This "Con-loss" test was carried out for the above 17 positive clones, and 4 of which were "Con-loss" positive, i.e., the ability of these yeast to tolerate Bax expression is attributable to the presence of a library cDNA. The ability of the remaining 13 clones to survive Bax killing is most likely due to mutations in the yeast strain and was not pursued further in this study. Plasmids were extracted from the 4 "Con-loss" positive clones and re-introduced into strain QX95001 to verify that these plasmids were indeed responsible for suppressing Bax-induced cell death in yeast.

Nucleotide sequences of the four positive clones were obtained and used to search against nucleotide sequence databases using the BLAST program (Altschul et al., 1990). Interestingly, three of the positive clones contain overlapping fragments of the same cDNA designated BI-1, for Bax-Inhibitor 1, and is essentially the same as the previously reported gene: TEGT (Walter et al., 1995). TEGT (Testis Enhanced Gene Transcript) was accidentally identified and the function of which was completely unknown (Walter et al., 1995). Suppression of Bax-induced yeast cell death by BI-1 is shown in Figure 2A. The fourth clone is a novel cDNA named BI-2. Thus, we have identified two human genes that inhibit Bax-induced cell death of yeast.

**BI-1 does not affect the production of Bax in yeast.** One potential mechanism by which BI-1 suppresses Bax-induced cell death in yeast is through inhibiting transcription from the *GAL10* promoter that drives the expression of *bax* cDNA in QX95001 cells. To exclude this trivial explanation, BI-1- encoding plasmid pQX36-1 (isolated from the HepG2 library) was co-transformed into cells of strain EGY48 that harbors a yeast plasmid containing *bax* cDNA under the control of the strong constitutive *ADH1* promoter (Honada et al., 1995). In this assay, BI-1 suppressed Bax killing despite the fact that Bax was expressed from a different promoter (data not shown), suggesting that BI-1 may not suppress Bax killing of yeast by preventing transcription of the *bax* cDNA.

To directly confirm that BI-1 does not prevent the production of Bax protein in yeast, Western blot analysis was carried out using cell extracts prepared from strain QX95001 (which express Bax under the control of the galactose-inducible *GAL10* promoter ) and BI-1 suppressor strain (harboring both the bax-containing and BI-1-containing expression plasmids) grown in glucose medium (also known as dextrose, D), and also 20-h after transferred to galactose medium (G). As shown in Figure 2B, Bax protein was produced at similar levels when yeast strains were grown in galactose-containing medium, regardless of whether cells contained the BI-1 plasmid. Therefore we conclude that BI-1 does not affect expression of the Bax protein in yeast cells and the reason that BI-1 suppresses Bax toxicity in yeast is most likely due to the ability of BI-1 to inhibit the pro-death function of Bax.

**BI-1 is an integral membrane protein.**

Despite no recognizable homology identified between BI-1 and any known protein, a Kyte-Doolittle plot of the protein hydrophobicity predicted 6-7 transmembrane domains. we also searched against a transmembrane protein database (TMbase) using a computer program called TMpred and the most favored transmembrane topology of BI-1 is shown in Figure 3A; BI-1 was predicted to have six transmembrane helices with both the amino (N) and carboxyl (C) termini facing the cytosol. The presence of multiple transmembrane domains and significant amount of hydrophobic residues suggest that BI-1 is likely an integral membrane protein.

To confirm that BI-1 is an integral membrane protein biochemically, we examined the detergent partitioning feature of BI-1 *in vivo*. It has been shown before that integral membrane proteins can be separated from hydrophilic proteins by a temperature-induced phase separation in the detergent Triton X-114 (Bordier, 1981). To this end, 293T cells were transiently transfected with plasmids containing either HA-tagged BI-1 or the vector alone. 2 days after transfection, BI-1-or vector- transfected cells were lysed in buffer containing Triton X-114. Total cell lysates were separated into the detergent-enriched and detergent-depleted (aqueous) phases essentially as described in Ausubel et al. (1991). Both phases were analyzed by SDS-PAGE and Western blotting. As shown in Figure 3B, BI-1 was mostly in the detergent-enriched phase, suggesting that BI-1 is an integral membrane protein. As a positive control, the integral membrane protein Bax also partitioned into the detergent phase. Therefore based on both the highly hydrophobic nature and detergent partitioning characteristics, BI-1 is most likely an integral membrane protein.

**Functional study of BI-1.**

**a. BI-1 inhibit Bax induced apoptosis in 293 cells.** Previously it was shown that transient transfection of Bax-encoding plasmids into the human embryonic kidney cell line 293 induces apoptosis (Zha et al., 1996). Using this transient transfection assay, we explored the function of BI-1 in human cells. Plasmid pcDNA3-hBax was co-transfected with equal amounts of pcDNA3 parental vector (used as a negative control), pcDNA3-BI-1-HA (HA-tagged BI-1), and pRC/CMV-Bcl-2 (used as a positive control for suppression of Bax-induced apoptosis). One day after transfection, both the floating and adherent cells were collected and subjected to the vital dye trypan blue exclusion assay. As shown in Figure 4A, BI-1 suppressed Bax-induced apoptosis in 293 cells, the degree of protection by BI-1 was comparable to that afforded by Bcl-2. In pcDNA3-hbax transfected cells, Bax was expressed at similar levels (Figure 4B), suggesting again that suppression of Bax-induced apoptosis by BI-1 was not due to downregulation of Bax expression. Expression of HA-tagged BI-1 protein and Bcl-2 was verified by Western blot analysis using an anti-HA antibody (12CA5) and anti-Bcl-2 antiserum (Figure 3B). We noticed that the HA-tagged BI-1 protein did not run uniformly in SDS gel, probably due to the highly hydrophobic nature of the protein. DAPI stain of the nuclei showed that Bax indeed induced apoptosis under these conditions (judged by the appearance of fragmented nuclei). These data suggest that in 293 cells, the function of BI-1 is similar to that in yeast cells in

that BI-1 inhibits Bax-induced cell death without interfering with the production of Bax protein.

**b. BI-1 inhibits serum withdrawal-induced apoptosis.** We also determined if BI-1 can affect apoptosis induced by physiological stimuli. Serum deprivation is commonly employed as a mean to induce apoptosis in mammalian cells, we therefore examined the consequence of BI-1 overexpression on serum withdrawal induced apoptosis using the human fibroblast GM701 cells. For this experiment, we used the green fluorescent protein (GFP)-tagged BI-1, because the BI-1-GFP fusion protein essentially retained the biological function of wild-type BI-1 in the 293 transient transfection assay (Figure 5A), furthermore, GFP-BI-1 functions somewhat better than the native BI-1, possibly due to higher stability of the fusion protein (Figure 4A and Figure 5A). GM701 cells were transiently transfected with either parental vector, pEGFP-BI-1 (GFP-tagged BI-1) or pRC/CMV-Bcl-2. A pact-βgal reporter plasmid was also co-transfected into cells as a marker. 18-h after transfection, cells were washed in medium containing 0.1% FBS and incubated in this medium for 24 hours. Floating cells were then collected and stained together with adherent cells using the chromogenic β-galactosidase substrate X-gal. Transfected cells (indicated by the blue color) were scored as either live or apoptotic based on their morphology under a light microscope; flat adherent cells were counted as live and small rounded cells or cells with blebbed membrane were counted as apoptotic. As shown in Figure 5B, BI-1 was able to inhibit serum-withdrawal induced apoptosis, the degree of protection was once again comparable to that by Bcl-2.

**BI-1 inhibits IL-3 withdrawal-induced apoptosis in lymphocytes.** In the above transient transfection models, BI-1 was able to inhibit either Bax or growth factor-withdrawal induced apoptosis. To further establish the role of BI-1 as an apoptosis inhibitor, I determined the function of BI-1 in the pro-B lymphocytes, FL5.12 cells. Bax was shown previously to promote IL-3 withdrawal-induced apoptosis in these cells (Oltvai et al., 1993). To this end, we stably transfected FL5.12 cells with either plasmid pEGFP-BI-1 or pRC/CMV-Bcl-2 together with a pBabe-puro vector that confers puromycin resistancy. Puromycin-resistant cells were selected and pools of stable transfectants were subcloned by limited dilution and subclones were screened for expression of the GFP-tagged BI-1 protein by fluorescence microscopy. Subclones that express Bcl-2 were screened by immunoblot analysis. BI-1-GFP polyclonal cells (BI-1), a stable clone of BI-1-GFP (BI-1-c12), a Bcl-2 stable clone (Bcl-2-c3), and control cells (harboring empty vector) were subjected to IL-3 withdrawal-induced apoptosis assay essentially as described (Oltvai et al., 1993). As shown in Figure 6A, polyclonal BI-1 transfectants were moderately resistant to IL-3 deprivation where as BI-1 clone 12 was significantly resistant to IL-3 withdrawal-induced apoptosis, although the Bcl-2 clone showed the strongest resistancy to IL-3 withdrawal. The expression levels of BI-GFP fusion protein was quantified by FACS analysis. As shown in Figure 6B, clone 12 displayed a uniform expression of BI-GFP, as manifested by a peak of green fluorescence (a significant shift from the negative control); whereas the green fluorescence from polyclonal BI-1 transfectants were heterogenous, but clearly a portion of the cells expressed the BI-1-GFP which account for the modest resistancy to IL-3 deprivation. Thus, BI-1 can protect FL5.12 cells from IL-3-withdrawal-induced apoptosis. These functional studies taken together suggest that BI-1 inhibits both Bax-induced and growth factor withdrawal-induced apoptosis.

**Tissue distribution of BI-1.**

The Rat homolog of BI-1/TEGT was cloned before and was found to express in all the rat tissue/organs examined (Walter et al., 1994). To further explore the expression

pattern of BI-1 in human tissues, Northern blot analysis was performed using poly(A)$^+$ RNA from eight different human tissues. As shown in Figure 7, BI-1 is expressed in all the human tissues examined, including heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas. Interestingly, two different size transcripts were detected for BI-1, with major mRNA of 2.8 kb and 1.0 kb. Similar size transcripts were identified for Rat and human *tegt* before (Walter et al., 1994, 1995). It remains to be determined if these different size transcripts arise from alternative splicing mechanisms and if they encode different proteins.

**BI-1 is localized to intracellular membranes.**

**a. GFP fusion study.** Bcl-2 and Bax are mostly in intracellular membranes, with majority of Bax in the mitochondria, whereas Bcl-2 is in the endoplasmic reticulum, nuclear envelope and mitochondria. To preliminarily explore the intracellular location of BI-1, BI-1 was expressed in several different cell lines (293, Cos-7, GM701) as GFP fusion, using the expression plasmid pEGFP-N2 which encodes a double mutant of GFP with brighter fluorescent properties than the wild-type GFP. This GFP-tagged BI-1 was verified to function as apoptosis inhibitor in 293 cells by a transient transfection assay as described above (Figure 5A). In all three cell lines, fluorescent microscopy demonstrated that BI-1 almost exclusively associated with intracellular membranes in a pattern typical of the endoplasmic reticulum (ER) and its continuity with the nuclear envelope (Figure 8A), although a small portion of BI-1 may be in Mitochondria, based on two color analysis using a mitochondria-specific dye, Mitotracker (Figure 8B).

**b. Indirect immunofluorescence.** We also confirmed the location of BI-1 by indirect immunofluorescence. Cos-7 cells were transiently transfected with an expression plasmid containing Flag-tagged BI-1. 24 hour after transfection, cells were seeded on chamber slides and immunofluorescence was performed using anti-Flag antibody M2. As shown in Figure 8A panel c, the BI-1-Flag fluorescence was essentially at the same location as GFP-BI-1, further indicating that BI-1 is located in intracellular membranes. Thus, BI-1 is primarily localized to intracellular membranes, similar to Bcl-2 family proteins.

**c. Subcellular fractionation.** We also explored the intracellular location of BI-1 by subcellular fractionation. For this purpose, 293T cells were transiently transfected with BI-1-HA containing plasmid or vector control. 2 days after transfection, cells were lysed in hypotonic buffer and separated into subcellular fractions essentially using the method described in Wang et al., 1996. As shown in Figure 9, BI-1 was mostly in the heavy membrane (HM) and nuclear (N) fraction (inferred as nuclear envelope based on the GFP fusion localization study). HM contains Mitochondria, lysosomes and some ER. F1-β-ATPase, PARP, and CPP32 were included as controls for Mitochondria, nuclear, and cytosolic fractions. Bax was present in both the heavy membrane (HM) and cytosolic (C) fractions in this fractionation procedure. Bcl-2 was found in the heavy membrane (HM) and nuclear (N, presumably nuclear envelope) fractions. Therefore by GFP fusion, indirect immunofluorescence and subcellular fractionation methods, BI-1 was found in the intracellular membranes. Most significantly, BI-1 co-localizes with Bcl-2 and to some extent also with Bax.

**BI-1 interacts with Bcl-2.**

**a. In vivo crossing linking.** The subcellular fractionation data suggest that BI-1 and Bcl-2 co-localize to intracellular membranes. To further address the question of whether BI-1 and Bcl-2 associate in the same complex, we carried out an *in vivo* cross-linking experiment. Plasmid containing either Flag-tagged or HA-tagged BI-1 was co-transfected with Bcl-2 into 293 cells. 2 days after transfection, cells were incubated with the thiocleavable chemical crosslinker DTBP. After crosslinking, cells were lysed and

immunoprecipitation was carried out using either the anti-Flag or anti-HA antibody. Crosslinked complexes were cleaved by β-Mecaptoethanol and Western blot analysis was performed using anti-Bcl-2 antiserum. As shown in Figure 10, either the HA-tagged or Flag-tagged BI-1 protein was cross-linked to Bcl-2, suggesting that BI-1 and Bcl-2 are most likely in the same complex. In the same experiment, Bax was not cross-linked to BI-1, suggesting that the cross-linking reaction was highly specific.

**b. Co-immunoprecipitation.** The observation that BI-1 can be cross-linked to Bcl-2 suggest that these two proteins are in close vicinity, but does not address the question of whether BI-1 physically interacts with Bcl-2. To answer this question directly, we determined if BI-1 and Bcl-2 can co-immunoprecipitate. For this purpose, we co-transfected Bcl-2 with either Flag-tagged BI-1 plasmid, or empty vector. (It should be noted that 293 cells have bearly detectable endogenous Bcl-2, we therefore boosted up the level of Bcl-2 by transfecting in a Bcl-2 plasmid). 2 days after transfection, cells were lysed in lysis buffer containing 1% NP-40 and immunoprecipitation was performed by diluting the extract to 0.5% NP-40. Either mouse immunoglobulin (mIgG1, as a negative control) or the anti-Flag monoclonal antibody M2 was used to pull down the BI-1-Flag immunocomplex. Western blot was then performed and probed with anti-Bcl-2 antiserum. As shown in Figure 7B, Flag-tagged BI-1 protein co-immunoprecipitated with Bcl-2, suggesting that these two proteins may indeed physically interact with each other.

Similar experiments were carried out to determine if BI-1 interacted with Bax and another pro-apoptotic protein Bak. In this case, 293 cells were co-transfected by BI-1-Flag with either Bax or vector, lysed and immunoprecipitation was carried out as described above. As shown in Figure 11, BI-1 failed to co-immunoprecipitate with either Bax or Bak. 293 cells have high endogenous Bak, and therefore we did not further increase the level of Bak by transfection. In conclusion, BI-1 interacts with Bcl-2 but not Bax or Bak.

Due to rapid development of the field, we have made some adjustment to the Statement of Work outlined in the proposal. Structure-function study of Bax (Aim#1 of proposal) has been accomplished and published (Zha et al., 1996). Results of this study provide additional evidence that the mechanism by which Bax kills mammalian cells and yeast may be similar, since the same functional domain of Bax (the BH3 domain) is crucial for the death-inducing activity of Bax in both yeast and mammals. Therefore, we felt more strongly that a functional screening in yeast (Aim#3 of the proposal) will identify mammalian genes relevant to the regulation of apoptosis in mammals, and Aim#3 of the proposal was put on top of the priority list. As described above, rapid progress has been made along this line of investigation. Because of these exciting findings, we plan to focus on the two new genes, BI-1 and BI-2, that we have identified. In addition, in collaboration with Dr. Shigemi Matsuyama (a postdoc in our lab), mutant screening in yeast has also been carried out (Aim#4) and a number of yeast mutants have been identified that inhibit Bax-induced yeast cell death. We are in the process of characterizing these yeast genes.

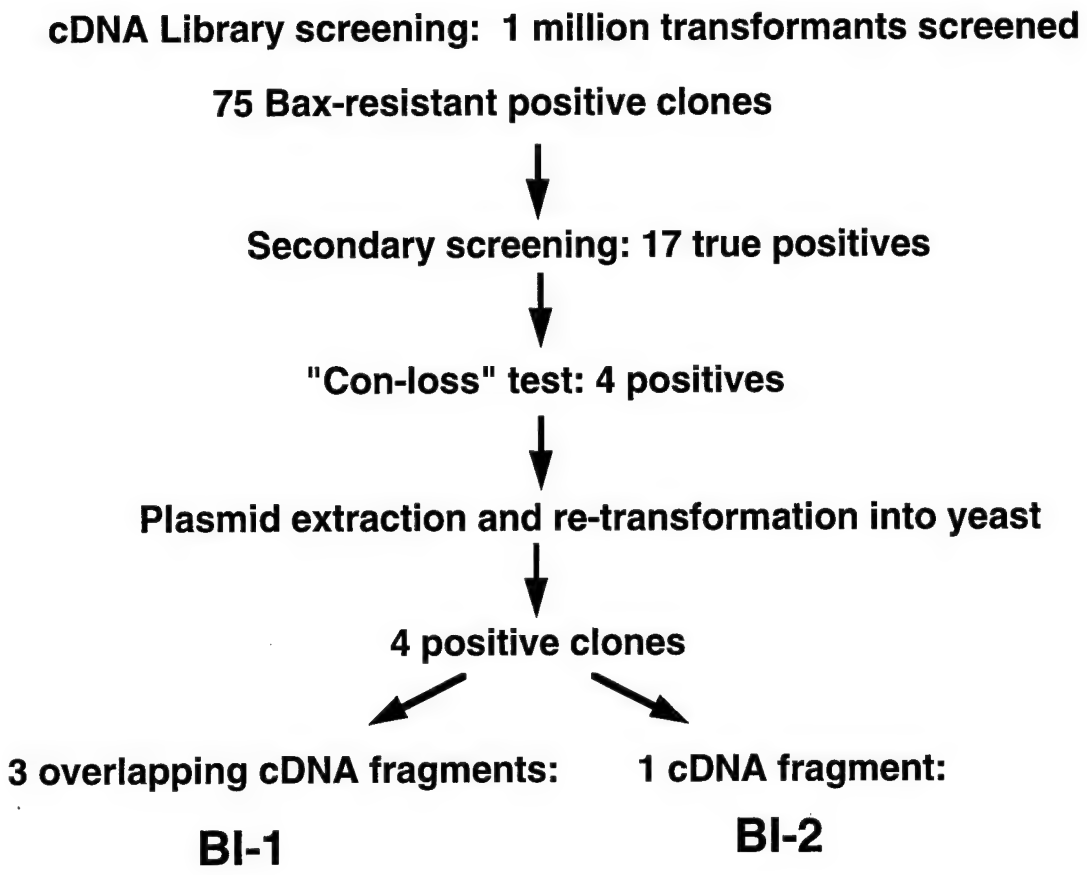


**Figure 1. Library Screen for Inhibitors of Bax-induced Lethality**

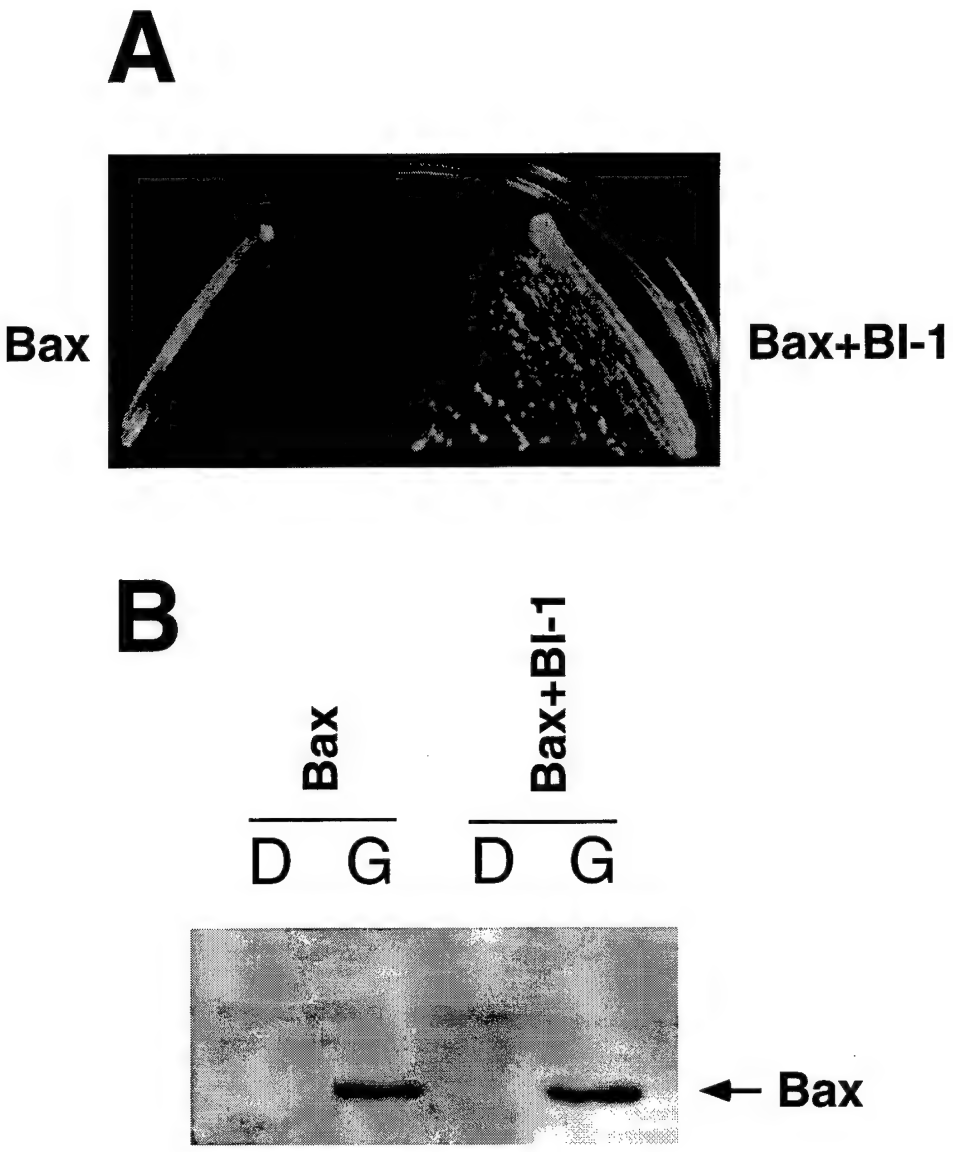


**Figure 2. BI-1 suppresses Bax-induced yeast cell death.**

Either control vector or BI-1-encoding expression plasmid (isolated from the HepG2 library) was transformed into cells of yeast strain QX95001 (harboring Yep51-bax). (A) Transformants were streaked on galactose-containing synthetic medium lacking uracil and leucine. Photograph was taken after a 4-day incubation at 30°C. (B) Protein extracts were prepared from QX95001 transformants used in (A) which contained control or BI-1-encoding plasmids. Cells were grown in glucose-containing medium (D= dextrose) and then transferred to galactose-containing medium (G) for 20 h. Total protein extracts (20 µg/lane) were subjected to SDS-PAGE and immunoblot analysis using anti-mBax antiserum.

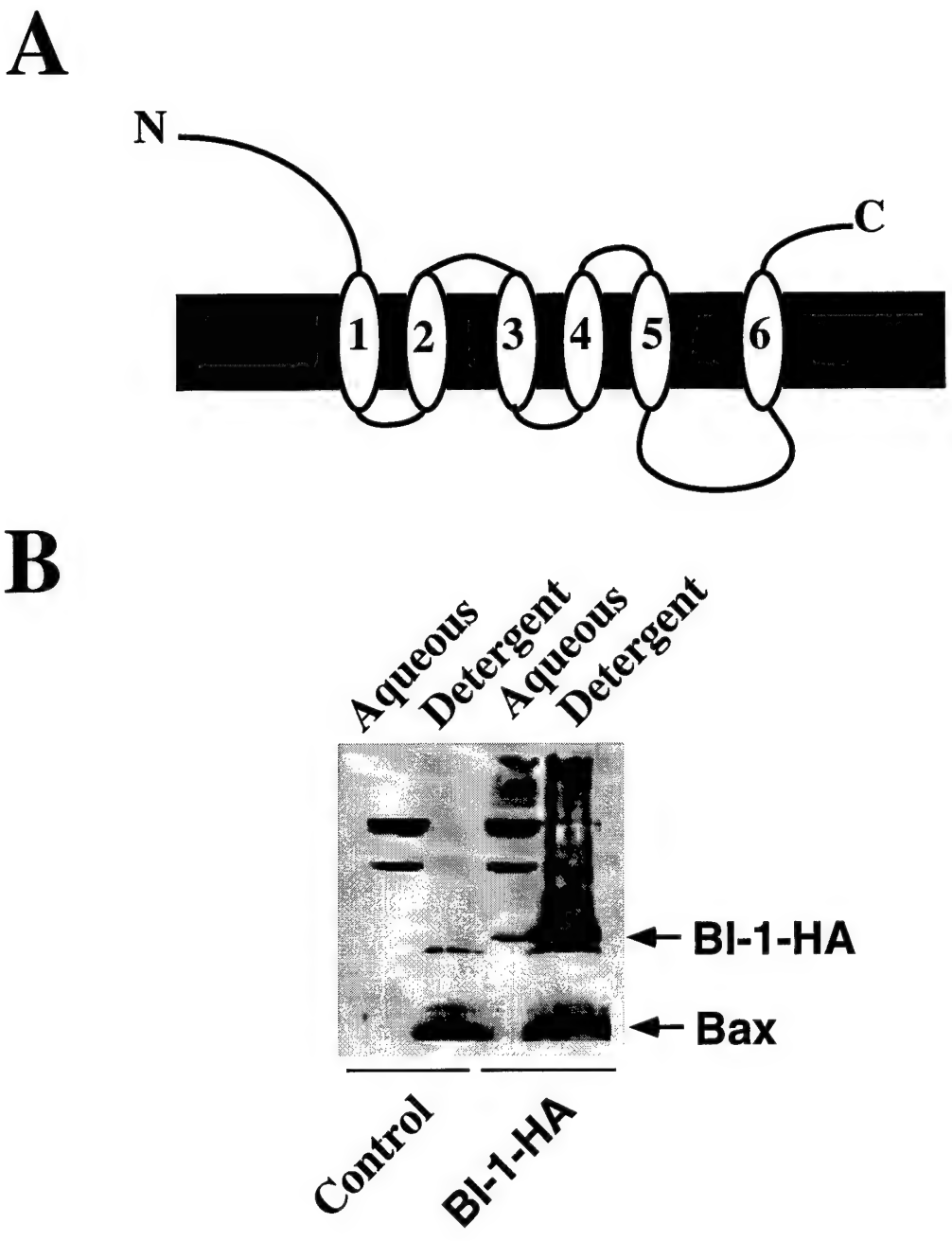


**Figure 3. BI-1 is an integral membrane protein.**

(A) Transmembrane topology of BI-1. The deduced BI-1 protein sequence was searched against TMbase (Transmembrane database) using the TMpred program for the prediction of potential transmembrane helices. The most favored model for the transmembrane topology of BI-1 is shown with six transmembrane domains. Filled black box represents lipid bilayer. (B) Triton-X114 partitioning of BI-1. A detergent phase partitioning experiment was performed using lysates from 293T cells that had been transiently transfected with either the control vector or HA-tagged BI-1-encoding plasmid as described in the Experimental Procedures. Equal portions of the aqueous (A) and detergent (D) fractions were subjected to SDS-PAGE/immunoblot assay using either anti-HA (top) or anti-Bax (bottom) antibodies as indicated.

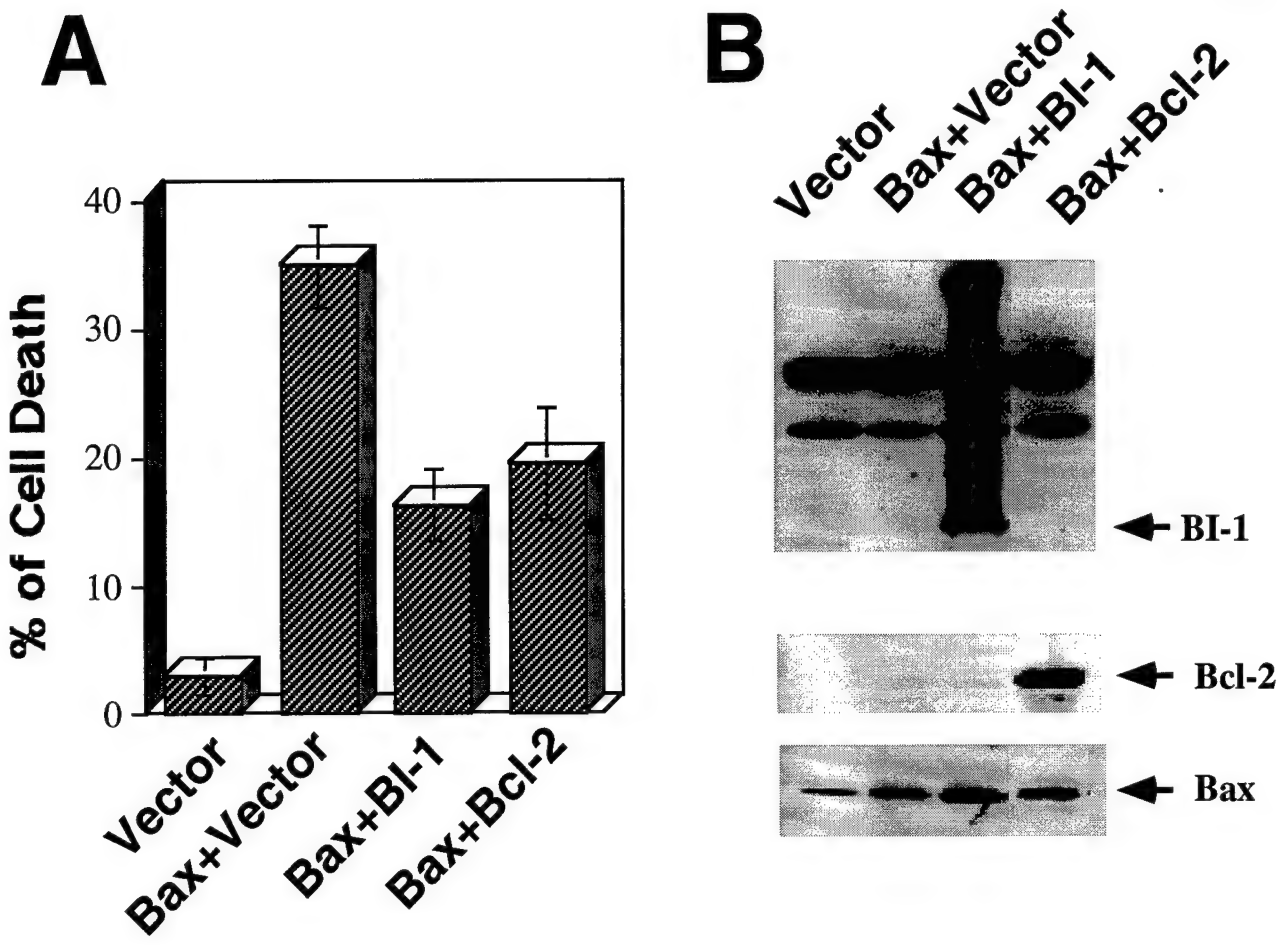


**Figure 4. BI-1 protects against Bax-induced cell death in 293 cells.**

293 cells were transiently transfected with the indicated plasmids (total 9 μg DNA for all transfections). 3 μg of bax plasmid was co-transfected with 6 μg of either control vector (pcDNA3) or plasmid encoding for BI-1 (with a C-terminal HA tag) or Bcl-2. One day after transfection, floating cells and adherent cells (after trypsinization) were pooled. (A) A portion of the pooled cells was subjected to vital dye trypan blue exclusion assay and at least 300 cells were assessed. Results shown represent mean ± SD from three independent experiments. (B) Extracts were prepared from another portion of the transiently transfected cells and subjected to SDS-PAGE/immunoblot analysis. The blot was sequentially probed with anti-HA monoclonal antibody, anti-hBax antiserum and anti-Bcl-2 antiserum. The blot was stripped after each antibody detection. The predicted band corresponding to the ≈30 KD BI-1-HA protein is indicated with an arrow. The origin of the slower migrating anti-HA reactive material presummably arises from the extremely hydrophobic nature of BI-1.

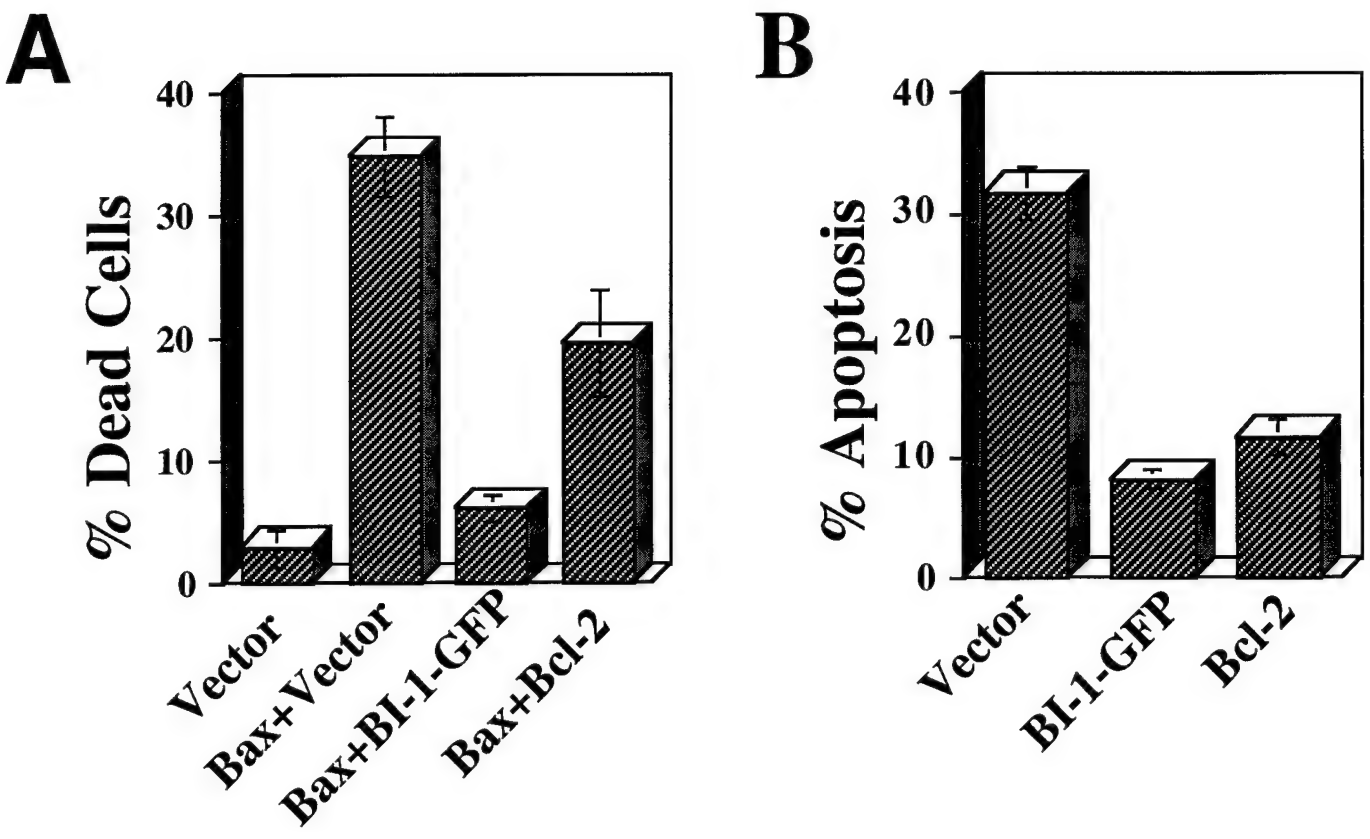


**Figure 5. BI-1 Protects against serum withdrawal-induced apoptosis in GM701 cells**

(A) GFP-tagged BI-1 retains the biological function of BI-1. 293 cells were transiently transfected with either vector control (9 μg) or co-transfected with 3 μg bax plasmid and 6 μg of either BI-1-GFP or Bcl-2 plasmid. % of trypan blue dye positive cells was determined one day later (mean ± SD, n=3) as described in Figure 3. The expression of GFP-tagged BI-1 was verified by fluorescence microscopy.
(B) BI-1 protects against serum-withdrawal induced apoptosis in GM701 cells.
GM701 cells were co-transfected with a β-gal reporter plasmid (0.5 μg) and the indicated plasmids (4.5 μg each). 18-h after transfection, cells were washed in DMEM containing 0.1% FBS and incubated in the same low serum medium for another 24 hours. Floating and adherent cells were fixed and stained with X-gal. The % blue cells (transfected) with apoptotic morphology was determined (mean ± SD, n=3).

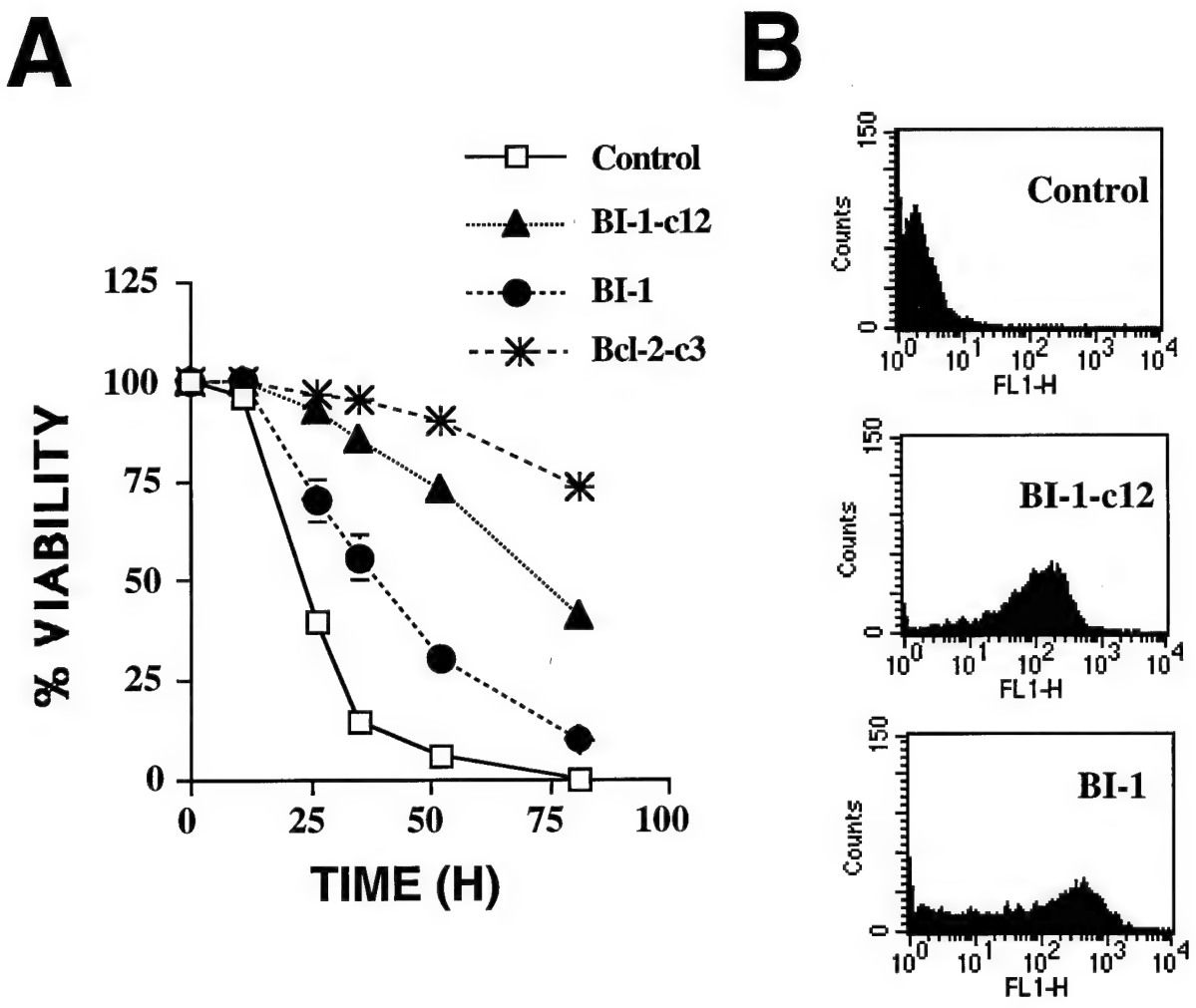


**Figure 6. BI-1 inhibits IL-3 deprivation induced apoptosis in FL5.12 cells.**

(A) Inhibition of IL-3 withdrawal-induced apoptosis. Stably transfected cell lines expressing GFP-tagged BI-1 or untagged Bcl-2 were created as described in the Experimental Procedures. Independent clones were obtained by limiting dilution for BI-1 (clone 12) and Bcl-2 (clone 3). Cells were grown to a density of ~5x105 cells/ml before removing IL-3 from the medium. At various times thereafter, samples were removed and subjected to trypan blue dye exclusion assay (mean ± SD; n=3).

(B) Expression of GFP-tagged BI-1 assessed by FACS analysis. Stably transfected FL5.12 cells containing either a negative control plasmid (pcDNA3) (top panel) or plasmid encoding BI-1-GFP were analyzed by FACS. The hitograms are presented for clone 12 which express BI-1-GFP at high levels (middle panel) and the polyclonal line prior to subcloning (bottom panel).

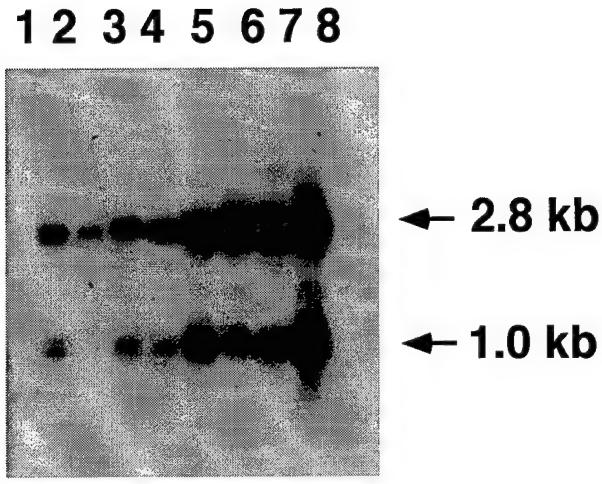


**Figure 7. BI-1 is ubiquitously expressed.**

Human poly (A)+ RNA derived from various tissues was analyzed by Northern blotting using a 0.7-kb fragment of BI-1 cDNA as a probe. Lanes 1-8 contain, in order, mRNA from human heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas.

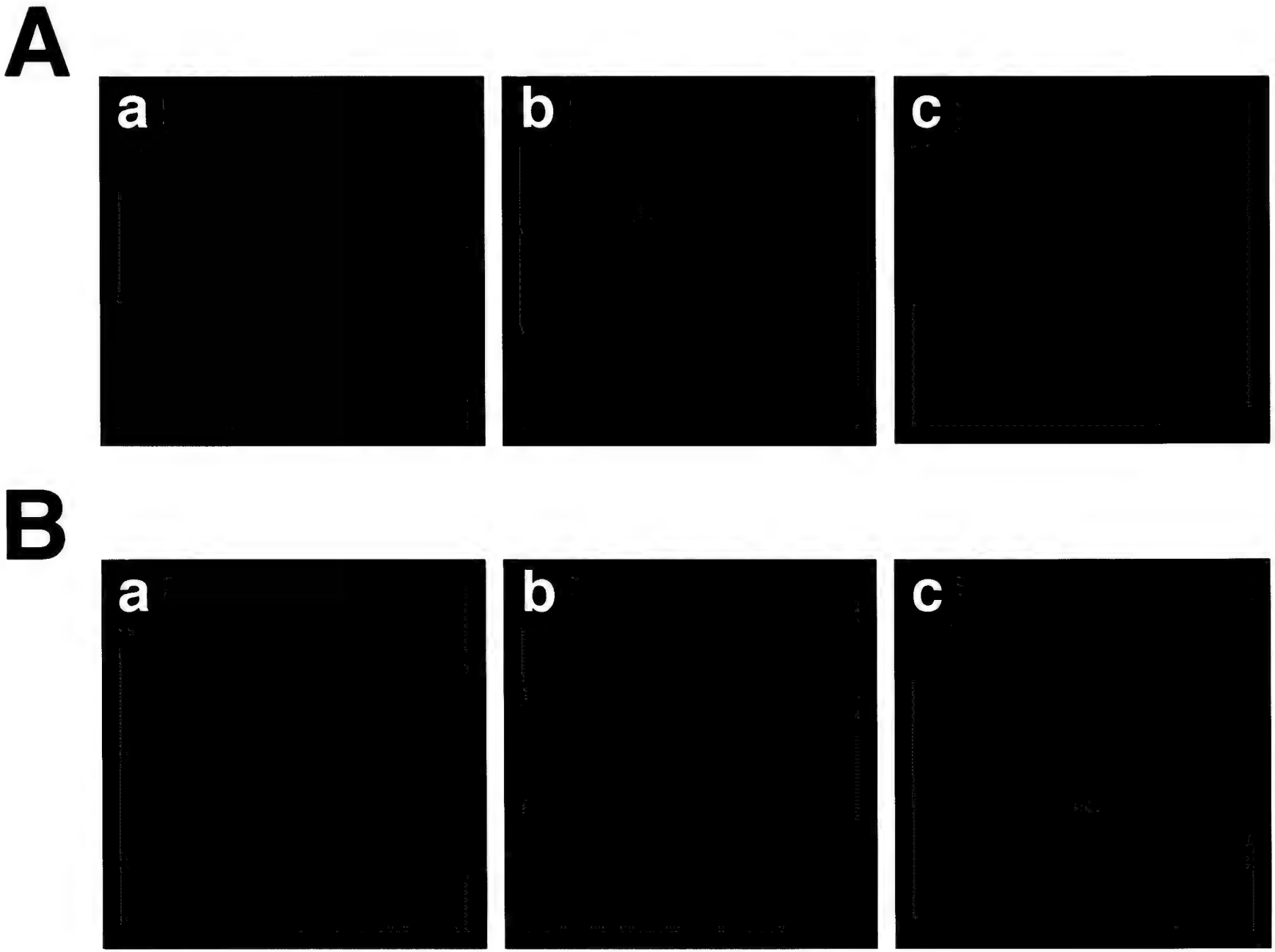


**Figure 8**

## Figure 8. BI-1 is localized to intracellular membranes.

(A) Either the parental pEGFP-N2 vector (a) or plasmid encoding BI-1-GFP fusion protein (b) or a plasmid encoding Flag-tagged BI-1 protein (c) was transfected into Cos-7 cells. 18-h after transfection, cells were seeded in chamber slides for fluorescence microscopy. In panel a and b, cells were analyzed directly using appropriate filters for visualization of the green fluorescence resulting from GFP. In panel c, cells were stained with anti-Flag M2 and FITC-conjugated anti-mouse IgG. Cells stained with secondary antibody alone exhibited negligble fluorescence (not shown). Photographs represent ~400x original magnification.
(B) BI-1-GFP transfected Cos-7 cells were incubated with the Mitotracker dye before being fixed and visualized by fluorescence confocal microscopy using filters appropriate for the visualization of green (a), red (b), or both (c), resulting from the BI-1-GFP protein and the Mitotracker. Data shown are representative of the majority of doubly stained cells.

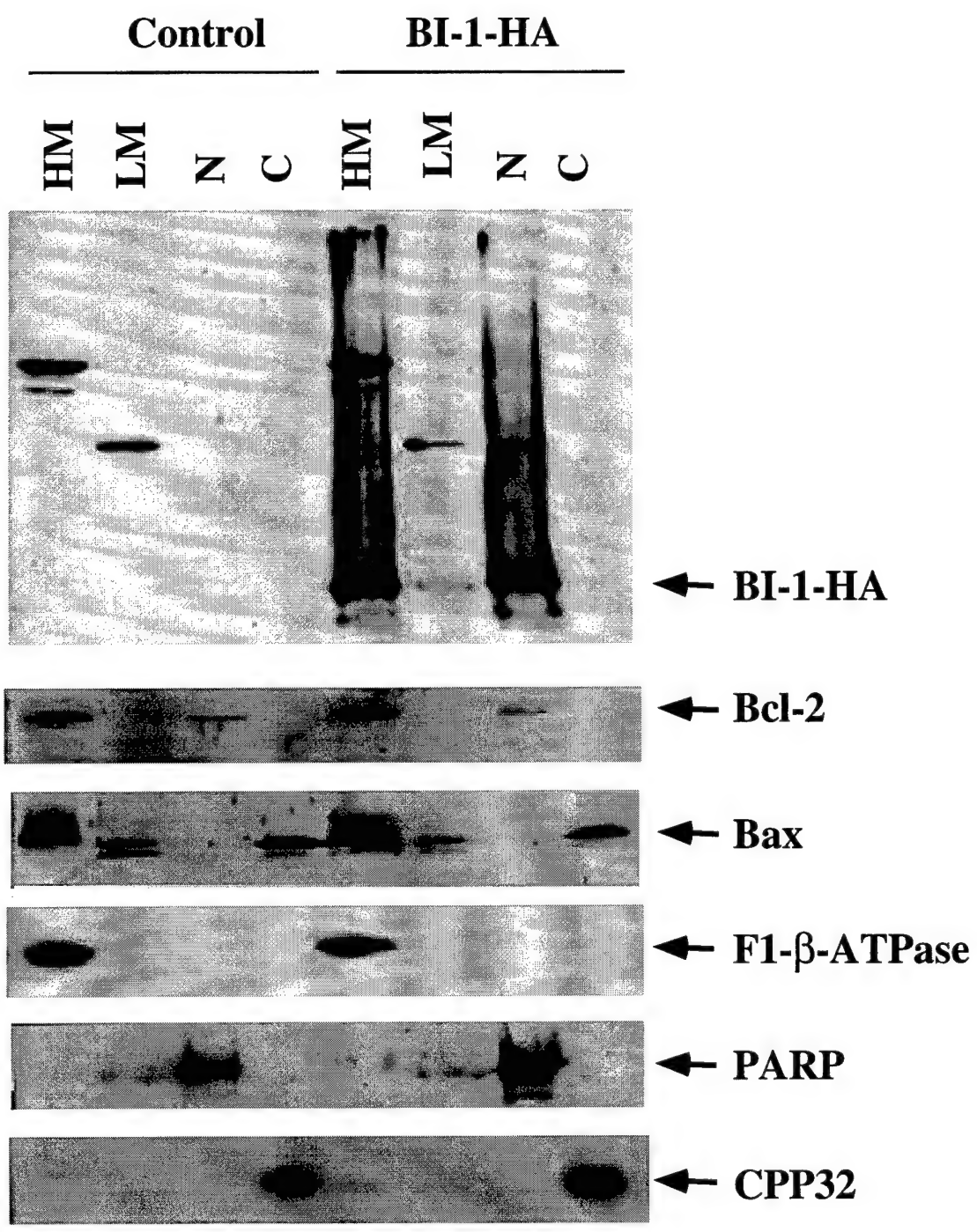


**Figure 9. BI-1 co-fractionates with Bcl-2.**

293T cells were transiently transfected with either parental vector (Control) or plasmid encoding HA-tagged BI-1. 2 days after transfection, cells were lysed in hypotonic buffer and crude subcellular fractionation was performed by centrifugation to produce Heavy Membrane (HM), Light Membrane (LM), N, Nuclear (N), and Cytosolic (C) fractions. Equivalant portions of each fraction were subjected to SDS-PAGE/immuno analysis using antibodies HA-tag, Bcl-2, Bax, F1βATPase (mitochondria marker), PARP (nuclear marker), and CPP32 (Caspase-3, cytosolic marker).

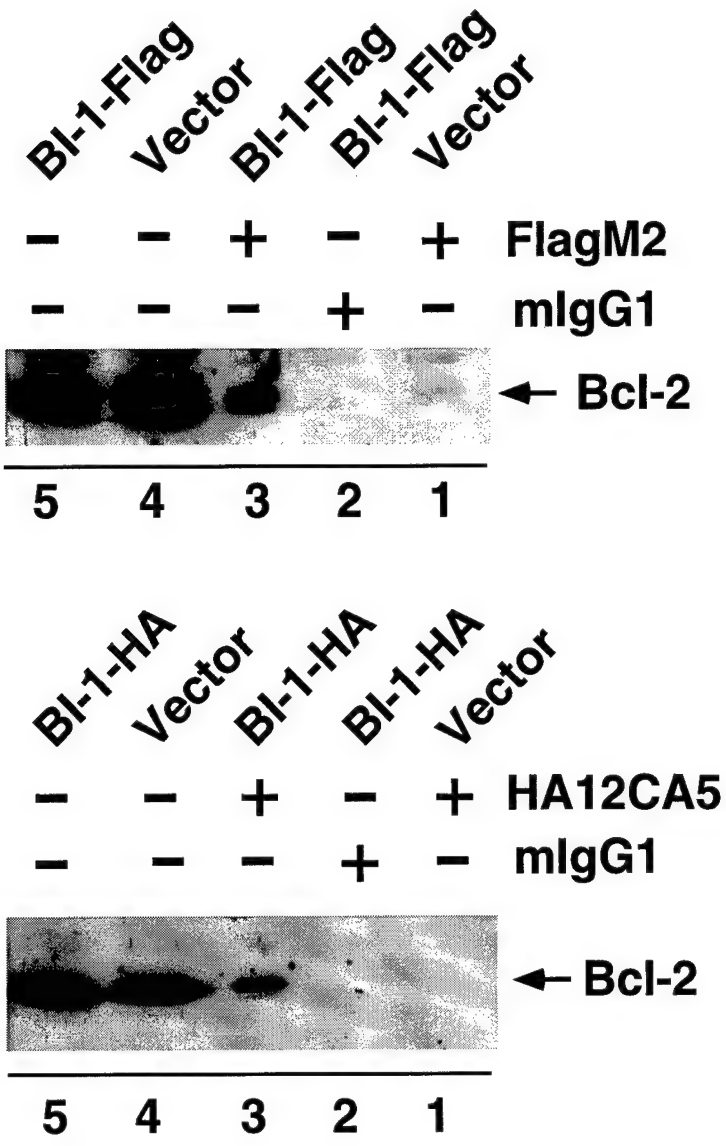


**Figure 10. BI-1 can be crossed to Bcl-2 in vivo.**

(A) *in vivo* cross-linking. 293 cells were co-transfected with Bcl-2-encoding plasmid and either a control (vector) or plasmids encoding either BI-Flag (top panel) or BI-1-HA (bottom panel) proteins. 2 days after transfection, cells were washed in PBS and incubated with the membrane permeable chemical crosslinker DTBP. After cross-linking, cells were washed in PBS and lysed in RIPA buffer. Immunoprecipitations were performed using normal mouse IgG as a negative control or the anti-Flag M2 (top) or anti-HA 12CA5 (bottom) monoclonal antibodies. Immunocomplexes were reduced (to reverse the cross-linking reaction) and denatured by boiling in SDS sample buffer containing 10% β-Mercaptoethanol. Immunoblot analysis was carried out using anti-Bcl-2 antiserum. Lanes 1 and 2 represent whole cell lysates from cells transfected with Bcl-2+BI-1 or Bcl-2+vector, respectively (1/20 of the input for lanes 3-5). Lanes 3-5 were loaded with immunocomplexes precipitated with the indicated antibodies.

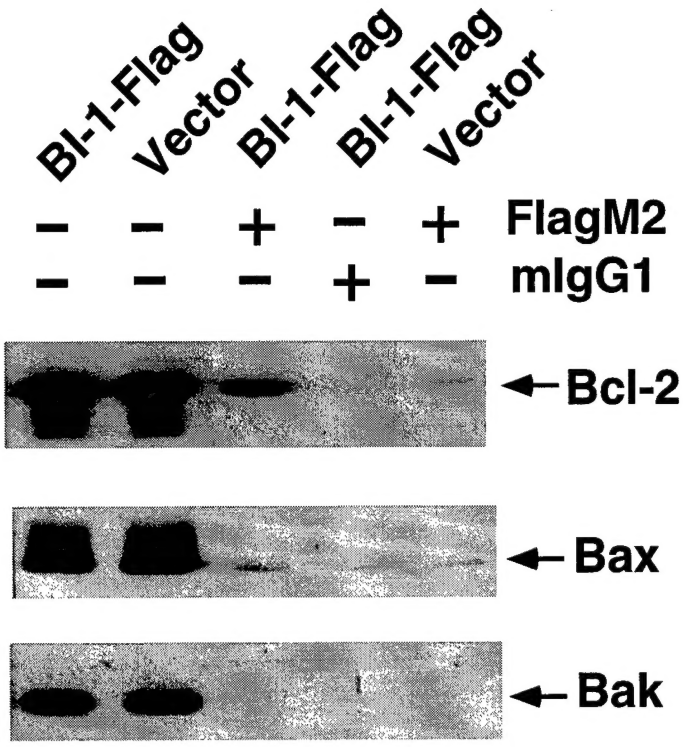


**Figure 11. BI-1 interacts with Bcl-1 in vivo.**

293 cells were transiently transfected with either Bcl-2- or Bax- encoding plasmids (top and middle panel, respectively) together with either vector control plasmid or BI-1-Flag-encoding plasmid DNA. 2 days after transfection, cells were lysed in 1% NP-40 buffer and immunoprecipitations were performed using either anti-Flag antibody M2 or normal mouse IgG. After extensive washing, immunocomplexes were boiled in SDS sample buffer and subjected to SDS-PAGE/immunoblot analysis using antisera specific for Bcl-2 (top), Bax (middle) or Bak (bottom). Lanes 1 and 2 represent whole cell lysates from cells transfected with Bcl-2 (top), Bax (middle), or no plasmid (bottom) together with either BI-1-Flag plasmid (lane 1) or vector control (lane 2), representing 1/40th the input for immunoprecipitation. Lanes 3-5 were loaded with immunocomplexes.

## (7) CONCLUSIONS

By performing a functional screening in yeast, we have identified two human genes that protect yeast cells against Bax induced death. BI-1 has been characterized in greater details. BI-1 is an integral membrane protein with six transmembrane helices. Similar to Bcl-2, BI-1 functions to inhibit apoptosis in mammalian cells and BI-1 is localized to intracellular membranes. More interestingly, BI-1 physically interacts with Bcl-2. Therefore, we have identified a novel apoptosis inhibitor functioning in the Bcl-2/Bax programmed cell death pathway.

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